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14. ABSTRACT

We have completed all proposed research, except a part of Task 4-related to xenograft tumor experiments in nude mice. This will be completed during no-cost extension period. We observed in this study that estrogen induced redox signaling mediates proliferation and growth of MCF-7 cells exposed to estrogens. Over-expression of biological ROS scavengers (MnSOD and Catalase) or treatments of cells with chemical antioxidants, [N-acetylcysteine (NAC) and ebselen], inhibits estrogen induced expression of cell cycle genes as well as prevention of estrogen-induced growth of malignant breast epithelial cells. Also, findings of this study support ROS functioning as signal molecules in E2-induced cell transformation. These findings suggest that, in addition to the estrogen receptor activity, E2-generated mitochondrial ROS may promote susceptibility to malignant transformation as well as growth of malignant breast cancer cells. Thus our results suggest: 1) a new paradigm that estrogen-induced mitochondrial oxidants control the early stage of cell cycle progression and growth of breast cancer cells, 2) estrogen-induced mitochondrial oxidants control cell transformation and invasiveness of transformed cells; and 3) provide the basis for the discovery of novel antioxidant-based drugs or antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer.

15. SUBJECT TERMS
Estrogen, oxidants, antioxidants, breast cancer

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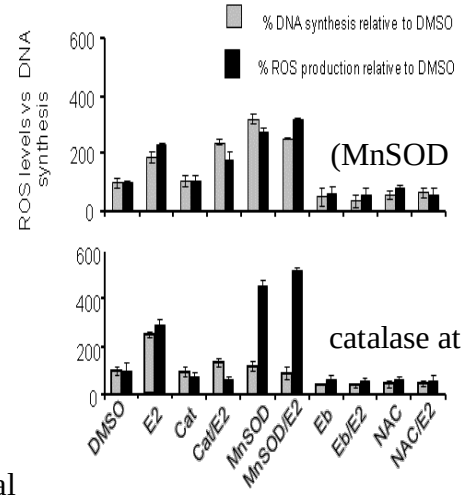
Final Report

Introduction: There is considerable evidence, both experimental and epidemiologic, that estrogens play a role in carcinogenesis; however, these effects cannot be fully accounted for by the mitogenic effects induced by estrogen stimulation of their receptors (ER) (Roy et al, 2004). Estrogens are known to promote proliferation and growth of estrogen responsive breast cancer cells through receptor and non-receptor mediated pathways. Estrogens have also been reported to increase intracellular reactive oxygen species (ROS) in breast cancer cells and other cell types. Low levels of ROS have been reported to induce proliferation, growth and metastasis of breast cancer cells and other cell types, irrespective of estrogen receptor (ER) status. In this application, we had proposed to investigate the role of estrogen-induced mitochondrial (mt) oxidant signaling pathways in the *in vivo* progression of breast cancer as a new line of research that may lead to the discovery of novel antioxidant-based drugs or new antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer. We proposed to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, *i.e.*, estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle.

Body

We have completed proposed research in the original First Task (i) both antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as, catalase or PrxIII; and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA by real-time RT-PCR, the rate of DNA synthesis by BrDu incorporation, and different phases of cell cycle by flow cytometry. We have also completed proposed research in the original Task (ii) by determining the morphology and behaviors of cells that over-express mtTRX2, mtSOD, mtPrxIII, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. Task (iii) of cell transformation was completed by determining: (a) Foci Formation, (b) Anchorage-independent (soft agar) cell growth, and (c) tumor spheroid formation using new 3D HuBiogel bioassay to test whether estrogen-induced conversion of normal cells to transformed cells is inhibited by treatment with antioxidants, over expression of MnSOD, catalase, PrxIII, Trx2; or mtTFA silencing. The task (iv) of growth of breast cancer cells was determined using (a) in vitro tumor formation (anchorage-independent cell growth), and (b) tumor spheroid formation using new 3D HuBiogel bioassay to test whether estrogen-induced conversion of normal cells to transformed cells is inhibited by treatment with N-acetylcysteine and ebselen; overexpression of MnSOD, catalase, PrxIII, Trx2, or mtTFA silencing. Finally task (v) (no cost extension) was tested in mice whether co-treatment with antioxidant, over expression of MnSOD, catalase, PrxIII, Trx2, or silencing of mtTFA prevents growth, angiogenesis, and invasion of tumors. Major findings are described in detail below:

1. **Treatment with antioxidant as well as overexpression of catalase and MnSOD prevent estrogen-induced proliferation and growth of MCF-7 cells:** MCF-7 cells containing MnSOD and Catalase Adenovirus cassettes and overexpressing biological ROS modulators and catalase), were pre-treated with 40 μM ebselen or 1.0 mM NAC and then challenged with 100 pg/ml estrogensMCF-7 cells containing null vecotors were used as controls. Data reveals that treatments of cells with chemical antioxidants diminished ROS production and cell proliferation in a dose dependeent manner (Fig 1). It was also observed that cells expressing moi 50 diminished estrogen induced ROS production and increased DNA synthesis. MnSOD over-expression at moi 50 however, of increased estrogen induced ROS production as well as cell proliferation compared to estrogen alone treated groups (Fig 1A). At a higher 200 moi dose of adenoviral infections catalase diminished estrogen induced ROS production and DNA synthesis butMnSOD significantly increased ROS production. MnSOD infecteed cells alsosignificantly reduced DNA synthesis compared to cells



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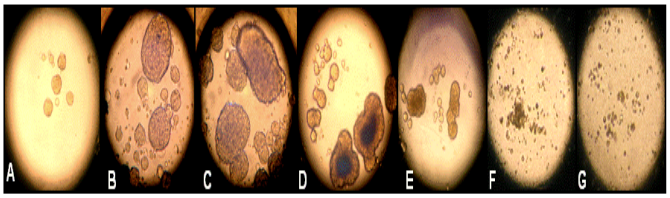
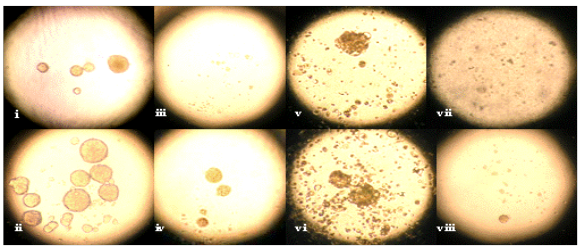
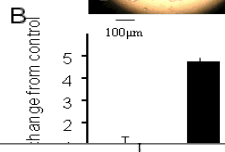


treated with moi 50 (Fig 1B). To resolve MnSOD contradictory data, we infected cells with various viral loads of MnSOD and treated with 100 pg/ml estrogens, then monitored growth characteristics on soft agar assay for three weeks. We observed that low dose of MnSOD (less than moi 50) induced sublethal ROS production and promoted growth of MCF-7 cells (Fig 2A-D) whereas viral load higher than moi 50 induced lethal dose of ROS and diminished cell growth (Fig 2E-G). Subsequent growth assay of cells infected with adenovirus expressing catalase or MnSOD at moi 100 or treated with chemical antioxidant (ebselen), inhibited estrogen induced growth without significant cell death (Fig 3). These data indicates that estrogen induced ROS mediates whether cell grow or not and that antioxidant treatments of cells inhibits estrogen induced growth of MCF-7 cells in vitro.





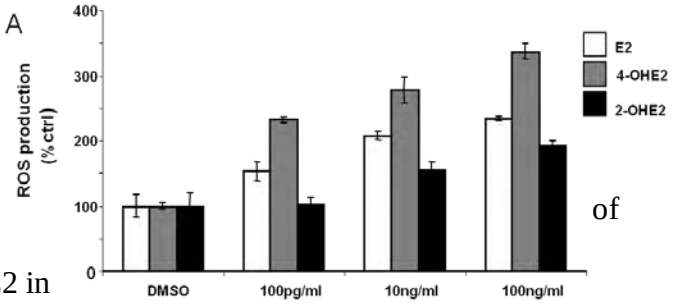
2. E2-induced mtROS modulates G_1 to S transition and through a nongenomic, ER independent signaling pathway: We investigated some of the early G1 genes whether 4-OH-E2-induced ROS signaling is involved in the transformation of normal breast epithelial cells to malignant cells. Using a normal cell line (MCF-10A) that develop preneoplastic foci in response to 4-OH-E2 or E2, the expression of cell cycle genes, cyclin D, pcna, cdc25c, cdc2, nrf-1 and prc1 was measured by real time RT-PCR (Fig. 4). After exposure of 8h following two 48 hrs treatments with 4-OH-E2; we observed an approximately 1.14, 1.55, 1.57, 1.81 and 1.0 ddCt increase value in the RNA expression of prc1, cdc2, nrf-1, cdc25c, and TFAM respectively. Overexpression of MnSOD markedly decreased 4-OH-E2 induced cdc2(-.45), cdc25c(-.86), and TFAM(-.39) expression compared to normal cells.

Sample	Detector	Avg Ct	dCt	ddCt	fold increase	relative ma
dmso 18 hrs	pcna	21.735	7.456	0	1	10
e2 100pg	pcna	21.654	6.991	0.47	1.38	14
MnSOD+dmso	pcna	22.354	7.79	0	1	10
MnSOD+E2	pcna	22.413	7.925	-0.14	0.91	9
catalase+dmso	pcna	21.588	7.412	0	1	10
catalase+E2	pcna	22.075	7.566	-0.15	0.9	9

Sample	Detector	Avg Ct	dCt	ddCt	fold increase	relative ma
dmso 3 hrs	cyclin D1	29.362	15.083	0	1	10
e2 100pg	cyclin D1	29.121	14.458	0.62	1.54	15
MnSOD+dmso	cyclin D1	30.103	15.539	0	1	10
MnSOD+E2	cyclin D1	29.708	15.221	0.32	1.25	13
catalase+dmso	cyclin D1	28.531	14.355	0	1	10
catalase+E2	cyclin D1	30.045	15.536	-1.18	0.44	4

Sample	Detector	Avg Ct	dCt	ddCt	fold increase	ve mRNA cc
dmso 18 hrs	pcna	35.139	20.86	0	1	10
e2 100pg	pcna	35.089	20.426	0.43	1.35	14
Ebselen+dmso	pcna	34.976	20.412	0	1	10
Ebselen+E2	pcna	34.732	20.244	0.17	1.12	11
NAC+dmso	pcna	32.927	18.751	0	1	10
NAC+E2	pcna	33.463	18.954	-0.2	0.87	9

3. E2-induced ROS formation in normal breast epithelial cells: Before carrying out cell transformation, we characterized normal human mammary epithelia cells for their ability to produce ROS in response to 17 beta-estradiol (E2) exposure. These cells respond to E2 in terms of producing ROS very similar to breast cancer cells. ROS production by E2 and its metabolites, 2-OH-E2 and 4-OH-E2 in normal human mammary epithelia cells was dose-dependent (Figure 5).



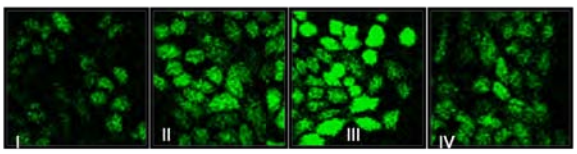


Figure 5. To determine whether estrogens and catechol metabolites induces ROS formation in normal human mammary epithelial cells, we seeded MCF-10A cells in 96well plates at 10k/well. 24hrs post seeding, medium was replaced with serum free media and cultured for 48hrs. Post starvations, DCFH-DA were diluted 1:1 in Pluronic® F-127 (20% w/v) after which it was further diluted in HBSS media at 10uM conc. DCFH-DA were loaded into each well and incubated for 20mins. DCFDA

solutions were removed and replaced with estrogens and metabolites diluted in serum free media, ROS were measured on Tecan Genios microplate reader and by microscopy, respectively. Data is presented here as ROS productions with values set to controls at 100% (mean of 3-4 experiments ± SD).

4. Dose-dependent colony formation by E2 in MCF-10A cells: We used the anchorage independent growth (AIG) assay to examine cell transforming ability of E2 by detecting AIG positive colony formation. E2 exposure to MCF-10A cells produced dose dependent increase in colony formation (Fig.6).

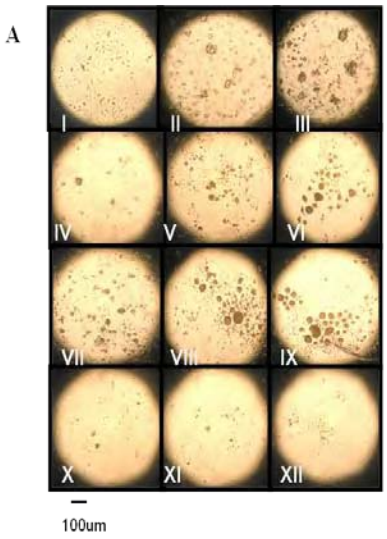
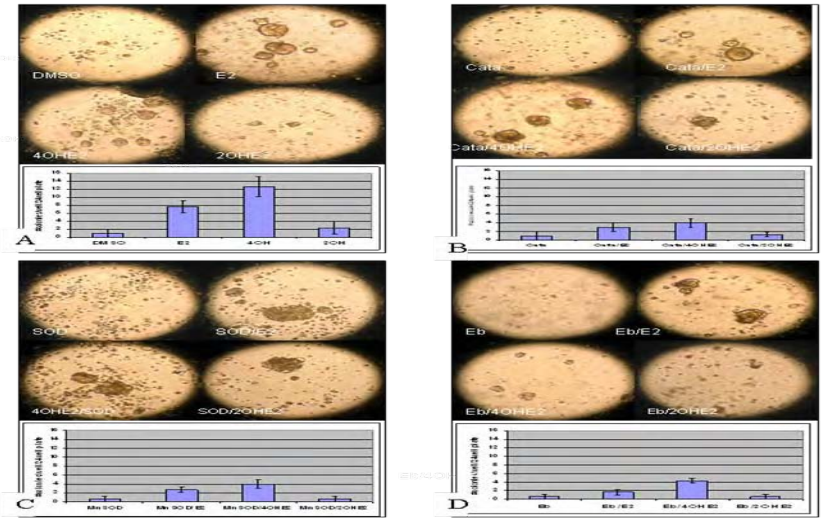


Fig 6. Representative picture of E2-induced MCF-10A transformed cells (left panel) and the bottom panel indicates the number of colony formed with different doses of E2 and its metabolites. Cells were seeded in 6 well plates and treated with 100pg/ml, 10ng/ml and 100ng/ml dose ranges of estrogens and catechol metabolites to determine transformation abilities of these estrogens. A. I) DMSO, II-III) 5.0 and 50 ug/ml BaP as positive controls respectively. We used estrogen concentrations of 0.1, 10.0 and 100.0 ng/ml respectively for transformation experiments; IV-VI) E2; VII-IX) 4-OHE2 and X-XII) 2-OHE2 treatments. B) Fold differences in colony formations were compared to colonies from 0.1ng/ml estrogen treatments. Each data represents mean of four wells +/- SD. Experiment were conducted four times and colonies formed in soft agar assays were enumerated as #colonies/well/24well plate (2cm^2 area) in quadruplicates wells.

5. Inhibition of E2-induced colony formation by ROS modifiers: In cells overexpressed with adenvirus construct containing catalase and MnSOD that lowers oxidant production as well as in mtTFA silenced cells, E2 produced fewer colony compared to E2 alone (Figure 7). Antioxidant ebselen also inhibited E2-induced cell transformation.

Figure 7 . To determine whether E2 induced mammary transformation is mediated by ROS, MCF-10A cells were co-treated with ROS modifiers. MCF-10A cells were infected with AdCMV-Catalase and AdCMV-MnSOD at moi 50 to overexpress these antioxidant enzymes. 48hrs post infections, chemical antioxidants were added to group and cells treated with 10ng/ml estrogenic compounds subject to transformation regimen. Colonies formed by E2 (A), is mitigated by over expression biological antioxidant catalase (B) or MnSOD (C). In addition, 40uM treatment with ebselen, a chemical antioxidant, also reduced numbers of colonies formed in mammary cells exposed to E2 or 4OHE2 (D). Experiments were conducted four times and colonies formed in soft agar assays were enumerated as #colonies/well/24well plate (2cm^2 area) in quadruplicates wells.



6. Clonogenic expansion of E2 transformed MCF-10A cells: To determine whether E2 induced colonies are clonogenic, we picked several colonies from each soft agar at the end of 21days and cultures them in media with 10% FBS, designated regular media (RM). Several of these clones did not passage beyond 10th passage in RM.

However, of the 5 that survived up to the 21st passage, we seeded these cells at 50 k cells/well in soft agar to determine whether these cells have acquired anchorage independent growth properties, a hallmark for transformed cells and whether these clones also respond to 100pg/ml estrogens. Cells were fed twice per week and cultured for 21days. Colonies were counted from quadruplicate wells, (± SD). One of our clones was highly clonogenic (P21) and responsive to E2 (Figure 8).

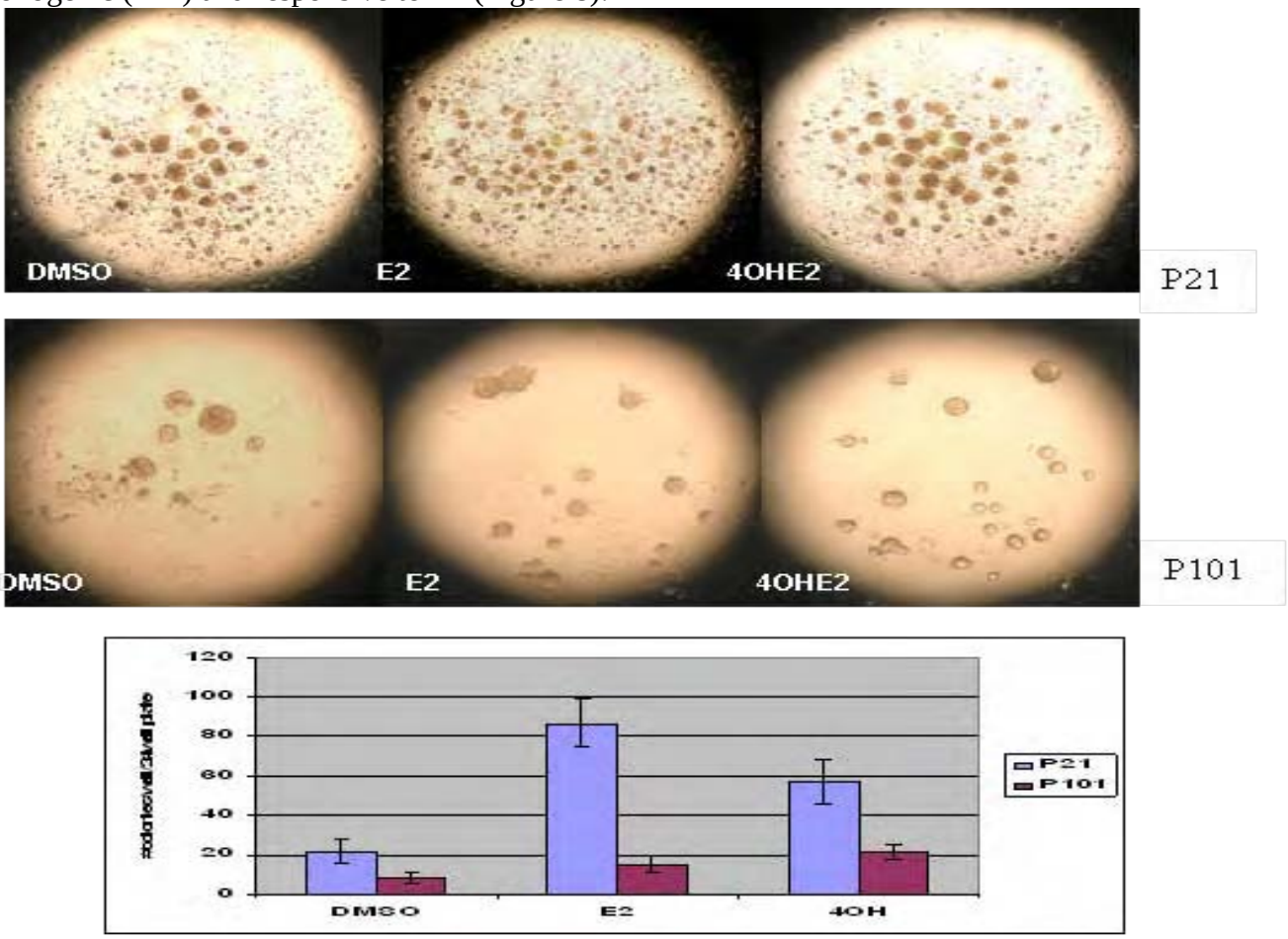
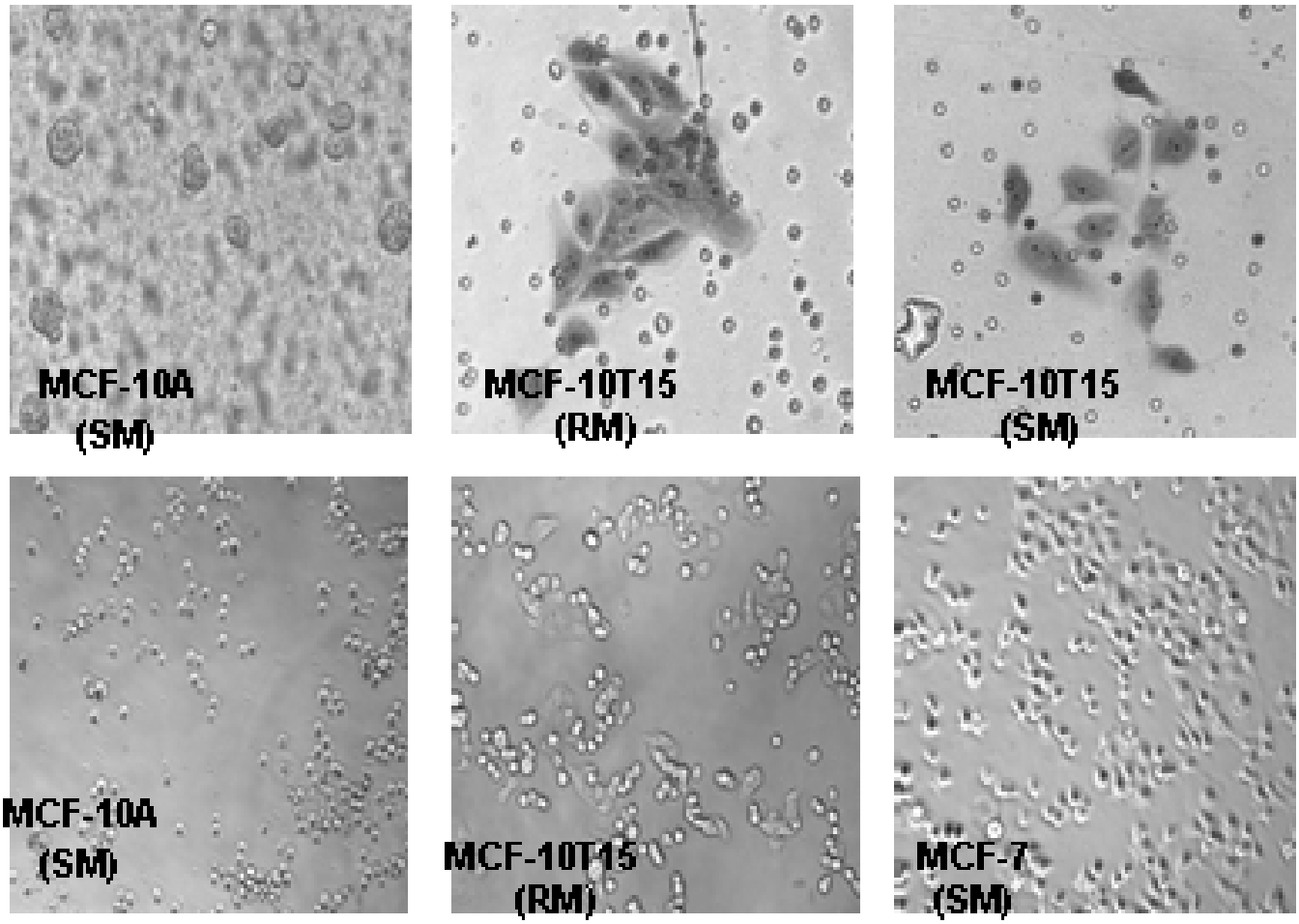
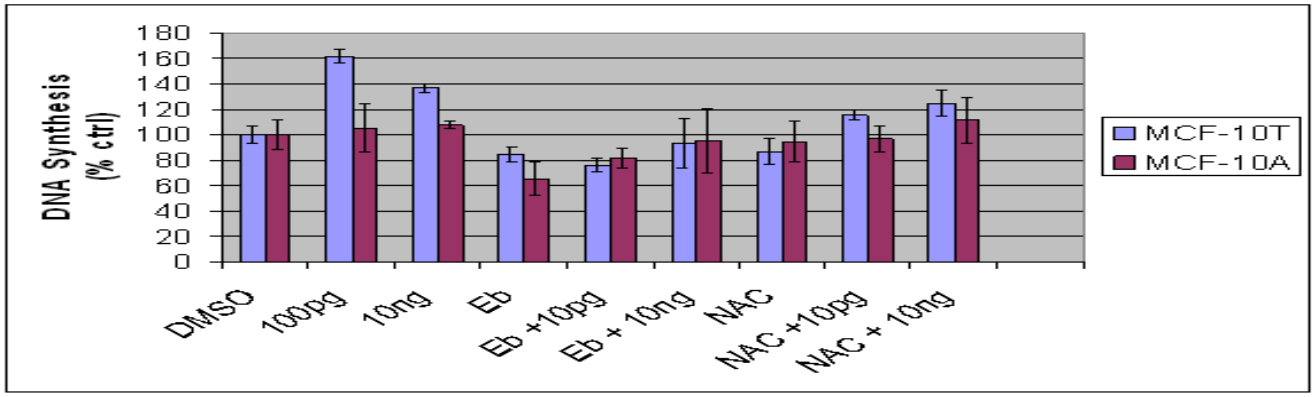


Figure 8. To determine whether growth of E2 induced transformed cells is estrogen dependent. MCF-10A transformed cells were treated with 10ng/ml estrogenic compounds subject to transformation regimen. Colonies formed by E2 were (A), is mitigated by over expression biological antioxidant catalase (B) or MnSOD (C). In addition, 40uM treatment with ebselen, a chemical antioxidants antioxidant, also reduced numbers of colonies formed in mammary cells exposed to E2 or 4OHE2 (D). Experiments were conducted four times and colonies formed in soft agar assays were enumerated as #colonies/well/24well plate ($2cm^2$ area) in quadruplicates wells.

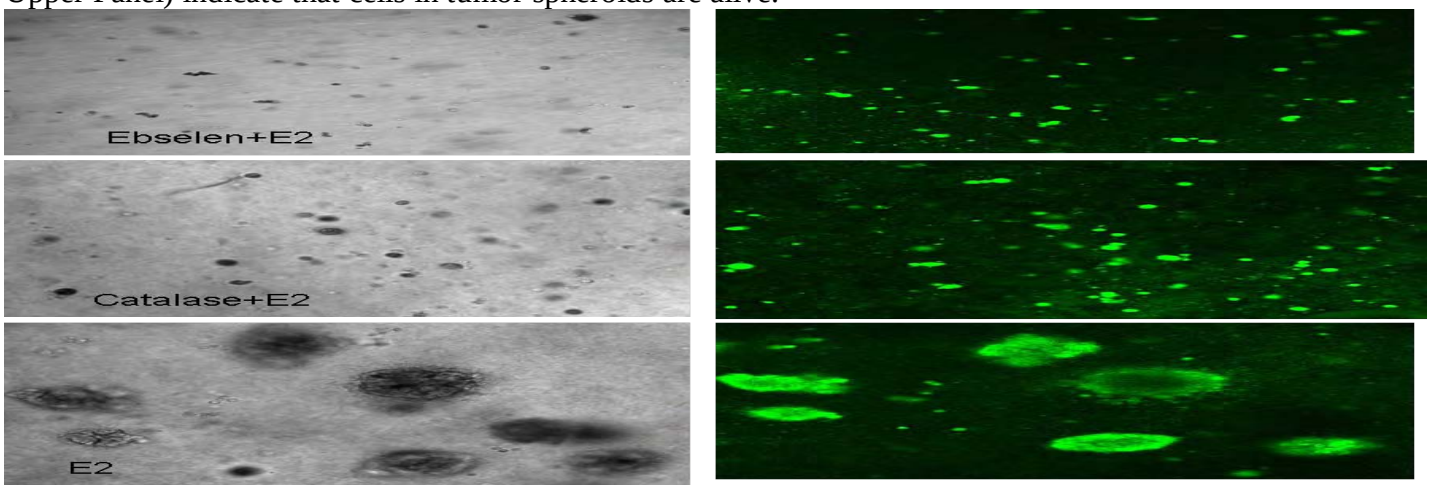
7. Analysis of invasive property of clone (MCF-10AT15) derived from E2 Induced transformation of MCF-10A: To assess invasive properties of the clones derived from E2 induced MCF-10A transformed cells, we seeded 10K MCF-10AT15 cells, a clone from MCF-10A transformation in BD BioCoat™ Matrigel™ Invasion Chambers. We also seeded these cells in glass chamber as we previously found that MCF-10A cells don't attach very well to glass in the first 16-24hrs. For the invasion assay (**a**), the chemotractant is either growth supplemented media (SM) or media with only 10% FBS (RM). MCF-10AT15 is highly invasive.



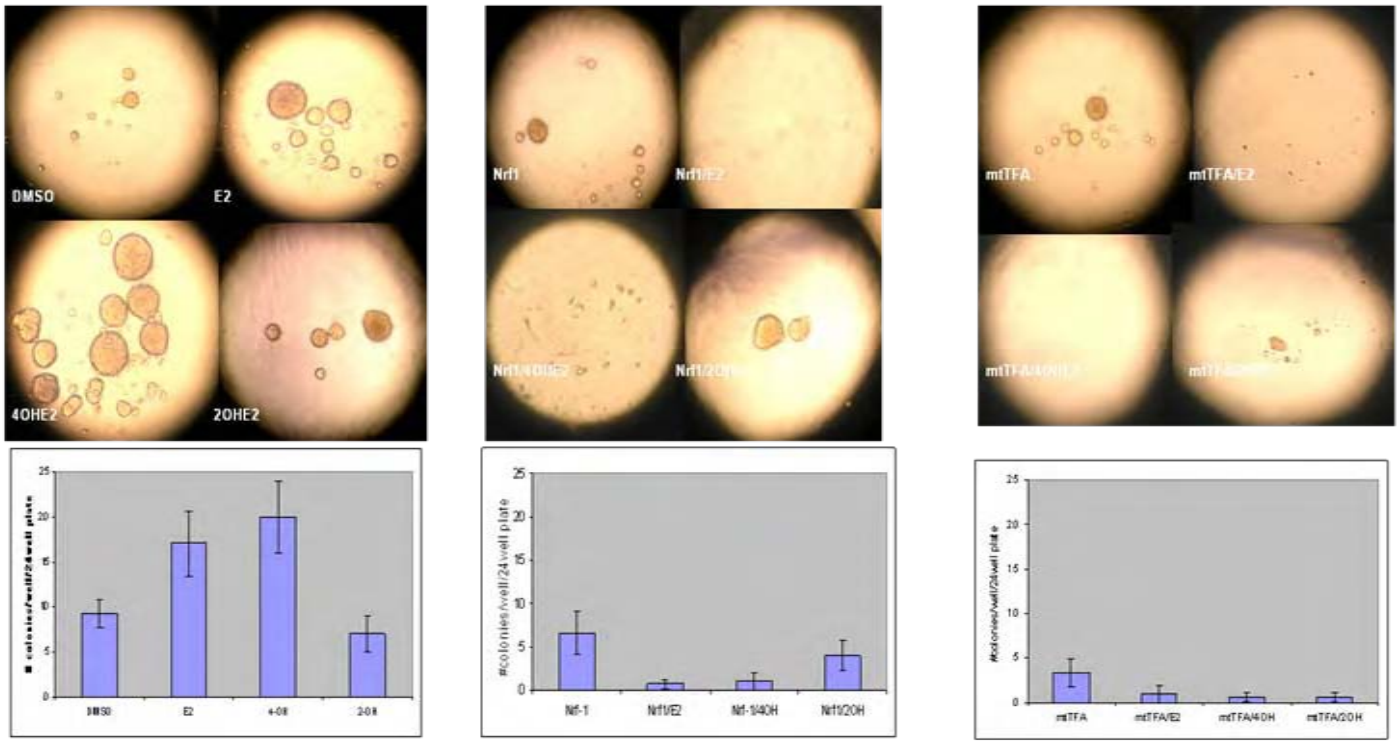
8. The growth of E2-induced transformed clone was highly responsive to E2 and was inhibited by both antioxidants, ebselen and N-acetyl cysteine. Antioxidants reduce E2-induced DNA synthesis in MCF-10A transformed cells. Cells ere grown in 96-well plates for 2 days in 10% FBS DMEM/F12 and serum starved 2 days prior to addition of E2 for 18 h-48 h unless specified otherwise. Bromodeoxy uridine (BrdU) incorporation assay was used to measure DNA synthesis as indicator of apoptosis in transformed cells. Antioxidants ebselen, NAC, and catalase were pretreated for 2 h prior to the addition of E2. Colorimetric BrdUrd incorporation was measured at 450 nm with a plate reader. Results are expressed as mean OD ± SD of three separate experiments with control set as 100% DNA synthesis. (**) Indicates treatment significantly different from control. (*) Indicates treatment significantly different from E2. (P<0.05).



9. <u>The E2-induced 3-D tumor spheroid formation was inhibited by ROS modifiers:</u> The tumorigenic conversion ability of E2-transformed MCF-10A cells was further investigated by 3-D culture using HuBiogel^{TM}. For 3-D culture, anchorage-independent MCF-10A human mammary gland epithelial cells transformed by E2 treatment were mixed with 3D HuBiogel^{TM} matrix containing DMEM-F12, seeded into 55 ml rotating-wall vessels and incubated at 37°C for 16 days. These conditions allow for the spontaneous formation of tissue-like spheroids. As shown in Fig 7 below, treatment with E2 produced spheroids (Fig.11 Left Upper Panel). Overexpreession of catalase and ebselen inhibited E2-induced tumor spheroid formation. Cells were labeled with CFSE using the Vybrant kit for checking viability. All spheroids showing the green fluorophore (Fig. 11 Right Upper Panel) indicate that cells in tumor spheroids are alive.



10. Silencing of NRF-1, a regulator of mtTFA and mtTFA (controls mitochondrial biogenesis) inhibited oxidant induced anchorage independent growth of MCF-7 cells: In NRF-1 (a regulator of mtTFA) or mtTFA RNAi silenced cells, E2 was not able to produce any colony. The effects of E2 on colony formation in cells transfected with scrambled RNAi of mtTFA or NRF-1 were similar to WT cells. This indicates that E2-dependent anchorage-independent growth of MCF-10A cells is dependent on ROS and mtTFA signalings.



11. Inhibition of estrogen-induced tumor formation by co- treatment with ebselen or over expression of antioxidant enzyme: The tumorigenic conversion ability of estrogen-transformed MCF-10A cells as described above in the section 4-6 was further investigated in nude mice to determine in vivo growth of malignant breast epithelial cells and to test whether attenuation of ROS formation by co-treatment with ebselen or overexpressing antioxidant enzymes, such as catalase, inhibits in vivo tumor formation. The nude mice were e inoculated sc with approximately 5 x 10^6 MCF-10A transformed with estrogen or transformed MCF-10A cells overexpressing catalase or treated with ebselen suspended in 0.3 ml Matrigel- MEM. Tumor volumes were monitored weekly by caliper measurement of the length, width, and height and were calculated using the formula for a semiellipsoid (4/3 r3/2). The tumor volume calculated using the following formula: length×width×height×0.5236 [Tomayko MM and Reynolds, 1989]. The estrogen-transformed cells formed tumor in nude mice (group 1 – estrogen transformed cells compared to group 6 –non transformed cells). Both treatments (overexpression of catalase and ebselen) delayed tumor formation of transformed cells shown in table below (Group 1 - 83% tumor compared to Group 2 – 16% tumor or Group 4 - 0% tumor). A two-way ANOVA analysis was performed to determine statistical significance with $p < 0.005$ considered significant (**). These results confirms in vivo the paradigm of chronic estrogen–induced ROS accumulation supports tumor formation, and also provide proof of principle that tumor formation by estrogen may be delayed or prevented by supplementation of antioxidant.

Xenograft Experiments in Nude Mice						
Groups	**Number**	**Cell Type**	**Animals with tumor**	**Treatment**	**Duration**	**%Tumor Incidence**
1)	6	MCF-10AET	5	E2	40 days	83
2)	4	MCF-10AETOvx	1	E2	40 days	16**
3)	4	MCF-$10AET^{Vector}$	4	E2	40 days	100
4)	4	MCF-1OAET Ebselen	0	E2	40 days	0**
5)	4	MCF-1OAET	2	DMSO	40 days	50
6)	4	MCF-10A	0	E2	40 days	0
Total Mice	**28**	ET=Estrogen Transformed, ETOvx= Estrogen transformed cells over-expressing catalase, Vector= Estrogen transformed cells with expression vector alone, Ebselen 20 uM, Tre atment = 0.125 mg E2/mice weekly once injected subcutaneously 3.5 ug. DMSO = Vehicle				

Key Research Accomplishments: Our findings provide evidence in support of the concept that 1) estrogen-induced mitochondrial (mt)ROS are involved in estrogen-induced in vivo growth of malignant breast epithelial cells. 2) Overexpression of mtSOD or catalase that detoxify mtROS, or cotreatment with the chemical antioxidant modifiers that reduces oxidants, prevents estrogen-induced cell transformation and in vivo growth of malignant breast epithelial cells. In addition to the estrogen receptor activity, *E2-generated mtROS may promote susceptibility to malignant transformation as well growth of malignant breast cancer cells both in vitro and in vivo.*

REPORTABLE OUTCOMES:

1. Abstract published in the Annual Meeting of Society of Toxicocology, 2007
2. Abstract submitted for Era of Hope meeting, 2008
3. Draft of the First Manuscript attached
4. Two additional Manuscripts are under preparation

CONCLUSIONS: We have completed all proposed research. We observed in this study that estrogen induced redox signaling mediates proliferation and growth of MCF-7 cells exposed to estrogens. Over-expression of biological ROS scavengers (MnSOD and Catalase) or treatments of cells with chemical antioxidants, [N-acetylcysteine (NAC) and ebselen], inhibits estrogen induced NRF-1 expression as well as prevention of estrogen-induced growth of malignant breast epithelial cells. E2 treatment to MCF-10A cells increased the formation of ROS. Over expression of biological ROS modifieers prevented E2-induced anchorage-independent growth of MCF-10A cells. We observed similar results with 3-D culture of transformed cells using HuBiogel. These data indicate that E2-induced mtROS are involved in the regulation of early G_1 phase progression and colony formation of breast cancer cells. Since neither antioxidants nor mitochondrial biogenesis blocker used in this study are reported to regulate the ER, our findings suggest that E2-induced mtROS modulates G_1 to S transition and some of the early G1 genes through a nongenomic, ER independent signaling pathway. Also, findings of this study support ROS functioning as signal molecules in E2-induced cell transformation. Thus, these findings suggest that, in addition to the estrogen receptor activity, *E2-generated mitochondrial ROS may promote susceptibility to malignant transformation as well as growth of malignant breast cancer cells. In summary,* our results suggest 1) a new paradigm that estrogen-induced mitochondrial oxidants control the early stage of cell cycle progression and colony formation of breast cancer cells; 2) estrogen-induced oxidants control cell transformation and invasiveness of transformed cells; and 3) provide the basis for the discovery of novel antioxidant-based drugs or antioxidant gene therapies for the prevention and treatment of estrogen-dependent breast cancer.

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APPENDICES-

1. Abstract published in Toxicologist as a part of the poster presentation in the annual meeting of Society of Toxicology, 2007

4-Hydroxy Estradiol-Induced ROS Mediated Activation of Cell Cycle Genes, CDC2 and Its Regulators CDC25C and PRC1, Controls Cell Transformation

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There is considerable experimental and epidemiological evidence that 4-hydroxy estradiol (4-OH-E2), a catechol metabolite of 17β estradiol (E2) plays a critical role in breast carcinogenesis; however, this effect cannot be fully accounted for by the mitogenic effects induced by estrogenic stimulation of its cognate receptor (ER). We have recently shown that reactive oxygen species (ROS) are involved in the regulation of E2 induced growth

of breast cancer cells. In this study we investigate whether 4-OH-E2-induced ROS signaling is involved in the transformation of normal breast epithelial cells to malignant cells. Using a normal cell line (MCF-10A) that develop preneoplastic foci in response to 4-OH-E2 or E2, the expression of cell cycle genes, cyclin D, pcna, cdc25c, cdc2, nrf-1 and prc1 was measured by real time RT-PCR. After exposure of 8h following two 48 hrs treatments with 4-OH-E2; we observed an approximately 1.14, 1.55, 1.57, 1.81 and 1.0 ddCt increase value in the RNA expression of prc1, cdc2, nrf-1, cdc25c, and TFAM respectively. Overexpression of MnSOD markedly decreased 4-OH-E2 induced cdc2(-.45), cdc25c(-.86), and TFAM(-.39) expression compared to normal cells. Overexpression of catalase also decreased 4-OH-E2-induced expression of prc1(-.50), cdc2(-.39), and nrf1(-0.4). We further examined whether nrf-1 interacts with potential binding sites that regulate transcription of those target cell cycle genes in cells exposed to 4-OH-E2. We found a greater rate of nrf-1 binding to chromatin in cells exposed to 4-OH-E2 compared to unexposed cells. In conclusion, these results indicate distinct altered expression of cell cycle genes following exposure to 4-OH-E2. We postulate that 4-OH-E2-induced ROS through nrf-1 mediated transcriptional activation of cell cycle genes, cdc2 and its regulators cdc25C (critical for mitotic entry) and prc1, controls 4-OH-E2-induced cell transformation. Supported by DoD grant BC05109

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Inhibition of Estrogen-Induced Growth of Breast Cancer Cells by Modulating In Situ Oxidant Levels

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The purpose of the our BCRP-funded proposal (BC051097) was to examine an untested and highly innovative concept in estrogen-induced carcinogenesis research, *i.e.*, estrogen-induced mitochondrial oxidants are involved in the promotion/progression of breast cancer through modulating signaling that controls the early G1 stage of the cell cycle. We previously reported that 17-β-estradiol (E2)-induced mitochondrial (mt) reactive oxygen species (ROS) act as signalling molecules. Here we have examined whether antioxidants, N-acetylcysteine and ebselen, overexpression of ROS lowering genes, such as, catalase and silencing of mtTFA are able to induce cell growth arrest in the presence of estrogen by analysis of the expression of early cell cycle biomarkers, cyclin D1 and PCNA by real-time RT-PCR, the rate of DNA synthesis by BrDu incorporation, and different phases of cell cycle by flow cytometry. We also determined the morphology and behaviors of cells that over-express mtSOD, catalase or silenced with mtTFA siRNA compared to those that do express these genes normally exposed to estrogen. Our data revealed that E2-induced cell growth was reduced by antioxidants N-acetyl-L-cysteine (NAC), catalase, and the glutathione peroxidase mimic ebselen. mtTFA siRNA transfection inhibited estrogen-induced proliferation of MCF-7 cells which is evident from the lower incorporation of BrDU in SiRNA treated cells compared to wild type cells in the presence of E2. We observed similar results by flow cytometery. In E2 treated MCF7 cells, the percentage of DNA content in S phase was 18% while this decreased to 6.8% in mtTFA silenced MCF7 after 24 h. The FACS data not only confirms the results shown by the BrdUrd assay, it also shows that impairment of mitochondrial biogenesis prevents E2-induced entry of MCF7 cells into the S phase by arresting them in the G0/G1 phase. Both antoxidant treatment and dextoxification of ROS prevented E2-induced expression of cyclin D1 and pcna, markers of cell proliferation detected by Real time PCR**.** In cells overexpressed with Adenvirus construct containing catalase that lowers oxidant production as well as in mtTFA silenced cells using their SIRNA, E2 was not able to produce any colony. Both antioxidants ebselen and N-acetylcysteine produced similar effects. It appears that E2 dependent colony formation rate of MCF-7 cells is dependent on ROS or mitochondrial signaling. Since neither antioxidants nor mitochondrial biogenesis blocker used in this study are reported to regulate the ER, our findings suggest that E2-induced mtROS modulates G_1 to S transition and some of the early G1 genes through a nongenomic, ER independent signaling pathway. These data indicate that E2-induced mtROS are involved in the regulation of early G_1 phase progression and colony formation of breast cancer cells. This work was performed under the BCRP-funded project BC051097 to DR.

3. Draft of the First Manuscript

Estrogen Induced Redox Signaling Mediates Proliferation And Growth of Breast Cancer Cells

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Abstract

Estrogens promote proliferation and growth of estrogen responsive breast cancer cells after binding to estrogen receptors (ER) and via non-ER mediated pathways. Estrogens have also been shown to increase intracellular reactive oxygen species (ROS) in breast cancer cells and other cell types which at a chronic sublethal levelhave been shown to induce proliferation,growth, and metastasis to cause therapeutic inconsistencies. The objective of the present workis to elucidate the mechanism(s) of estrogen-induced ROS mediated proliferation and growth of breast cancer cells. The study further examines whether biological, [Manganese Superoxide Dismutase (MnSOD) , Catalase],or chemical (ebselen or N-acetylcysteine) ROS modulators attenuate estrogen-induced growth in Human mammary adenocarcinoma cell line (MCF-7) . Our study reveals that Nuclear Respiratory Factor (NRF-1), a redox sensitive transcription factor known to regulate mitochondrial biogenesis, mediate survival and growth of MCF-7 cells during estradiol-17B (E_2) induced oxidative stress . Treatments of MCF-7 cells with either biological (Catalase and MnSOD) or chemical ROS modulator (Ebselen and N-acetylcysteine),which inhibits Akt phosphorylation orknocking down of NRF-1 expressions by siRNA prevented anchorage independent growth of cells. These findings suggest that estrogen-induced redox signaling of NRF-1 is critical for the in vitro growth of breast cancer cells.

Introduction

Estrogens are known to promote proliferation and growth of cancer cells via estrogen receptors (ER) and by the activation of non-ER pathways [4;5]. Metabolites of estrogens have also been implicated in impairing the tumor/suppressor gene functions [6]. Exposure to estradiol (also referred to as E2 or estrogen) in excess is often associated with breast, endometrial and ovarian carcinogenesis [1-3]. Recent studies have also indicated that estrogen induced ROS modulate proliferation and growth of breast cancer [7;8].

In vitro and in vivo studies have shown that malignant breast cells are under intrinsic oxidative stress when compared tothe normal breast cells This observation also positively correlates with an overload(?) of estrogen exposures [13;14] on the endogenous cellular antioxidant system [15]thereby causing inefficiencies in the processing of extra estrogen. Such disproportionate amounts of estrogen results in accumulations of oxidants such as hydrogen peroxide (H_2O_2) and hydroxyl ions ($-OH^-$) [16;17]. Chronic sub lethal levels of these oxidants can have significant consequences on the proliferation and growth of cancer cells as well as on apoptosis and cell death. [18-21]. While antiestrogenic compounds such as raloxifene and tamoxifen prevent growth of breast cancer cells by blocking ER activations, these compounds have also been reported to possess antioxidant properties [22-24]. Therefore, it is possible that the growth inhibitory properties of estrogen receptor blockers (antagonists??) also include reduction of ROS levels in breast cancer cells.

The complex mechanism involves cascade of phosphorylation and dephosphorylation and a number of signaling pathways e.g., the extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK), and the p38 kinases which culminates in the activation or deactivations of redox sensitive transcription factors [25]. Nuclear respiratory factor-1/alpha-palindrome-binding protein (NRF-1/alpha-PAL) is one such transcription factor that is up-regulated in response to oxidative stress) NRF-1 was initially found to mediate activation of the eukaryotic initiation factor 2a (eIF-2 alpha) [26] and cytochrome c expression [27]. However, its prominent role in retrograde communication between mitochondria and nucleus in regulating mitochondria biogenesis and oxidative phosphorylation (OXPHOS) has since been well established [28;29]. NRF-1 has also been shown to

promote survival and growth of cells during periods of oxidative stress [30]. Embryo lethality and depletion of mitochondrial content has also been shown inmice lacking NRF-1 [31]. Whereas estrogen exposure in malignant mammary cells up-regulate NRF-1 expressions [32] as well as promote growth of these cells [33], it is plausible that the growth promoting properties of estrogens in breast cancer cells are in part due to ROS regulated NRF-1 expressions.

In summary, our study reveals that estrogen induced redox signaling mediates proliferation and growth of MCF-7 cells exposed to estrogens. Over-expression of biological ROS scavengers (MnSOD and Catalase) or treatments of cells with chemical antioxidants, [N-acetylcysteine (NAC) and ebselen], inhibits estrogen induced NRF-1 expression as well as prevention of estrogen-induced growth of malignant breast epithelial cells.

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Materials and Methods

Cell Line and Cell Culture

MCF-7were obtained from American Type Culture Collection (ATCC) and were routinely cultured in phenol red-free DMEM/F12 media

(with 10% (vol/vol) FBS and , 100 μg/ml of Penicillin-Streptomycin .DMEM/F12 Media, FBS and antibiotics were purchased from Invitrogen corp, CA. For experimental purposes, cells were seeded at 10-75% confluence depending on the experimental designs and incubated CO_2 incubator with 85% humidity. Cells were allowed to adhere for 24 hrs.. after which culture medium were replaced with serum-free medium containing 100 μg/ml of Penicillin-Streptomycin, and cells were allowed to grow for another 48 hrs.. Serum deprivation allows cells to synchronize at G_0/G_1 phase of the cell cycle which were then treated with estrogens or antioxidants, as described in the figure legends.

Measurement of Reactive Oxygen Species (ROS)

Cellular ROS were measured as previously described [34]. Briefly MCF-7 cells were seeded at a concentration of 1.0×10^4 cells per well in black 96-well flat bottom plates (ThermoFisher Scientific Inc. USA) and allowed to adhere overnight. Cells were cultured for another 48 hrs. in serum free medium before a pretreatment for 4 hrs. with chemical antioxidants [ebselen and NAC]. diluted in Hank's balanced salt solution (HBSS), followed by incubation with 10 µM 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Invitrogen Corp, CA) for 20 minutes. DCFH-DA is a non-fluorescent cell-permeable compound, which is acted upon by endogenous esterases that remove the acetate groups generating DCFH. In the presence of intracellular ROS, DCFH is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF). DCFH-DA stock solutions were diluted at a 1:1 ratio with Pluronic F-127 (20% w/v). Cells were then rinsed with HBSS followed by various treatments with 100 pg/ml estrogens as described in the figure legends. The oxidative products were measured with a Tecan Genios microplate reader using 485 and 535 nm as excitation and emission filters, respectively.

RNAi transfections

Pre-designed and tetracycline inducible human shRNA for mitochondria transcription factor A (TFAM) and Akt_1 shRNA with corresponding null vectors were purchased from OriGene (OriGene Technologies, Inc. Rockville, MD). Pre-designed NRF-1 siRNA and corresponding scrambled constructs were purchased from Ambion (Applied Biosystems/Ambion, Austin, TX). shRNA for Akt_1, NRF-1 and TFAM were transfected into 35% confluent cells using FuGENE 6 (Roche) transfection reagents according to the manufacturer's protocol. Transfection efficiencies for all plasmid were determined by levels of each (AKt, NRF-1 and TFAM) of the protein expressions which ranged from 50% to 80% reductions in respective gene expressionsThe concentrations of plasmids used in all experiments were as recommended by manufacturer and were based on the sizes of multiwell plates used for experiments. After RNAi transfections cells were used for western blot analysis, BrdU and soft agar assays as described in subsequent sections.

BrdU cell proliferation assay

MCF-7 cells were seeded in 96-well plates at a density of 3,500 cells/well and incubated overnight. Cells were plated in quadruplicate for each experimental group. After 24hrs. cells were cultured for another 48 hrs. in serum free media . which were then treated with E_2 in the presence or absence of antioxidants for 18hrs.. Cell proliferations were measured by colorimetric immunoassay of BrdU incorporation into the DNA using Roche BrdU cell labeling kits as recommended by manufactures (Roche Molecular Biochemical, Indianapolis, IN). Briefly, cells were pulsed with BrdU labeling reagent for 3hrs. followed by fixation in FixDenat solution for 30 min at room temperature. Thereafter, cells were incubated with 1:100 dilution of anti- BrdU-POD for 1 hr. at room temperature to detect the immunoreaction by adding the substrate solution. Reading of the developed color was recorded at at 370 nm with a Tecan Genios microplate reader.

Adenovirus Infection

Adenoviruses cassettes containing MnSOD, Catalase for overexpression in MCF-7 cells or null vectors as controls were purchased from ViraQuest, Inc. (North Liberty, IA, USA). MCF-7 cells were seeded 24 hrs. in advance into appropriate dishes at 15-70% confluence depending on the experimental design. Cells were then infected with SOD, Catalase cassetes or null vector at 50 or 400 multiplicity of infections (moi) in serum free media. Cells were cultured for 48 hrs. before they they were used for experiments.

Chemical antioxidant treatments

The treatment procedure for ebselen (a glutathione peroxidase mimetic which also removes both H_2O_2 and peroxynitrite) and NAC (a precursor of glutathione and scavenger of ROS [35;36] differs according to the planned experiments. For example, in DCFH assays to measure intracellular ROS in live cells, 40 μM and 1.0 mM ebselen and NAC respectively were pre-loaded onto cells for2-4 hrs. For BrdU assays though, cells were treated with 40 μM and 1.0 mM ebselen and NAC respectively throughout the culture periods. For anchorage independent cell growth assays, antioxidants were mixed with cells and agar matrix before the assays, and added to media during bi-weekly feeding of colonies. For immunoprecipitations (IP) and western blot analysis, cells

were exposed to a single treatment with Ebselen and NAC during 48 hrs. of serum starvation periods before the western blot analysis.

Cell viability assay

CellTiter-Fluor™ Cell Viability kit were purchased from Promega corporation and used according to manufacturer's instructions. Briefly, cells were seeded in 96 well-plate at $1.0x10^4$cells/well, cultured in serum free media for 48 hrs. post seeding, and treated with estrogens and antioxidants. At the end, substrate reagent (GF-AFC) were mixed with buffer and dispensed into wells. This assay measures cell's protease activities in quiescent growth arrested cells at reduced mitochondrial activities when oxidant levels have diminished. Plates were read on fluorescence plate reader at 380–400 nm excitations and 505 nm emissions.

Western Blot Assays

Cells for western blot analysis were seeded in T-75 flasks and grown to 70% confluenceCells were harvested after the treatment as described in the plan in radioimmuno-precipitation buffer (150 mM NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (Roche, CA). Equal amounts of total cellular protein were mixed with loading buffer (25% glycerol, 0.075% SDS, 1.25% 2-mercaptoethanol, 10% bromphenol blue, 3.13% stacking gel buffer) and fractionated by electrophoresis on 12% polyacrylamide, 0.1% SDS resolving gels. Proteins were transferred to PVDF Immobolin-P transfer membranes (Millipore) using transfer buffer [25 mM glycine, 25 mM ethanolamine, and 20% methanol]. The membranes were blocked one hour at RT with blocking buffer [1×phosphate buffered saline, 0.1% Tween-20 with 5% (w/v) nonfat dry milk (PBS-T)]. Blots were subsequently incubated for 2 hrs. at room temperature with various antibodies as indicatedin figure legends.

Immunoprecipitation

In order to determine epigenetic modifications of NRF-1, cells were seeded in T-75 to achieve 70% confluence. Post treatments, cells were rinsed twice with 1x PBS, harvested with lysis buffer (150 mM NaCl, 0.5%

deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 50 mM Tris) containing protease and phosphatase inhibitors (Roche, CA). Whole cell lysates (WCL) were diluted to 500 µg of protein in 1 ml of lysis buffer, and samples were pre-cleared for 1 hr at 4 °C with 40 µl of a 1:1 slurry of protein A-agarose beads (Invitrogen Corp. CA) in lysis buffer and 1 µg of rabbit IgG. After a brief centrifugation to remove pre-cleared beads, 0.5 µg of anti-NRF-1 antibody was added to each sample and incubated on a rocking platform at 4 °C overnight. Captured proteins were pulled down by addition of 10 µl of protein A-agarose beads to each sample, and the slurries were incubated on the rocking platform at 4 °C for 2hrs.. The beads were then washed five times with lysis buffer and resuspended in 40 µL of 1×SDS electrophoresis sample buffer [50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol], resolved on 12% SDS-PAGE and transferred onto a PVDF nylon blotting membrane. To determine whether serine residues of NRF-1 are phosphorylated due to treatment, blot was probed with 1:5000 mouse monoclonal antibody against phosphoserine residue (Santa Cruz Biotech, CA). To determine the correlation between NRF-1 and TFAM in response to anti-estrogen treatments, WCL from same group were immunoprecipitated using rabbit anti TFAM and probed with mouse anti-TFAM. All primary antibodies were used at 1:1000 dilution and detected with horseradish peroxidase (HRP)-conjugated secondary antibody at 1:50,000 dilutions in PBS-T. ECL reagents (Amersham Biotech, ??) were used to develop the blots to detect the proteins by autoradiography.

Anchorage independent Growth

Anchorage-independent growth assays were performed as previously described [37]. Briefly, support agar for a base were made fresh by diluting 1.0% molten agarose at 42^{o}C in a water bath. Molten agar was then mix 1:1 with 2x culture media (2x phenol red free DMEM/F12 media made from powder, 20% charcoal/dextran stripped FBS, 2x Penicillin-Streptomycin antibiotics and 200pg/ml estrogens), then dispensed at 100 µl/well in 48 well plate and allowed to solidify for 4hrs. under the sterile hood. The top agarose overlay was made fresh by mixing 0.7% molten agarosemixed with 2x culture media and kept molten till needed in 42^{o}C. At the end, MCF-7 cells were harvested by trypsinisation and 5000-7500 cells/well were spun into pellet and resuspended with minimal

culture media. Cells were mixed with diluted top layer of molten agarose and carefully overlaid on the base agarose layer. The cells were incubated at 37°C in a humidified 5% CO_2 incubator. Cell cultures were fed once every week for a period of three weeks. Six replicate wells were used for each treatment condition and each experiment was repeated 3 times. Only formations of colonies of 100 microns or greater size were recorded by using metamorph software on Nikon 2000 E2. Images were acquired by Olympus C-5060 digital camera under microscope with 4x objective.

Statistical analysis

Results are expressed as mean ± S.D. Differences between means were evaluated by two-tailed Student's t-test. ANOVA was used to determine differences between groups.

Results

In vitro proliferation and growth of MCF-7 cells in the presence of estrogen induced ROS levels

MCF-7 cells containing MnSOD and Catalase Adenovirus cassettes and overexpressing biological ROS modulators (MnSOD and catalase), were pre-treated with 40 μM ebselen or 1.0 mM NAC and then challenged with 100 pg/ml estrogensMCF-7 cells containing null vecotors were used as controls. Data reveals that treatments of cells with chemical antioxidants diminished ROS production and cell proliferation in a dose dependeent manner (Fig 1). It was also observed that cells expressing catalase at moi 50 diminished estrogen induced ROS production and increased DNA synthesis. MnSOD over-expression at moi 50 however, of increased estrogen induced ROS production as well as cell proliferation compared to estrogen alone treated groups (Fig 1A). At a higher 200 moi dose of adenoviral infections catalase diminished estrogen induced ROS production and DNA synthesis butMnSOD significantly increased ROS production. MnSOD infecteed cells alsosignificantly reduced DNA synthesis compared to cells treated with moi 50 (Fig 1B). To resolve MnSOD contradictory data, we infected cells with various viral loads of MnSOD and treated with 100 pg/ml estrogens, then monitored growth

characteristics on soft agar assay for three weeks. We observed that low dose of MnSOD (less than moi 50) induced sublethal ROS production and promoted growth of MCF-7 cells (Fig 2A-D) whereas viral load higher than moi 50 induced lethal dose of ROS and diminished cell growth (Fig 2E-G). Subsequent growth assay of cells infected with adenovirus expressing catalase or MnSOD at moi 100 or treated with chemical antioxidant (ebselen), inhibited estrogen induced growth without significant cell death (Fig 3). These data indicates that estrogen induced ROS mediates whether cell grow or not and that antioxidant treatments of cells inhibits estrogen induced growth of MCF-7 cells in vitro.

Estrogen induced ROS activate Akt signaling pathway which mediates in vitro growth of MCF-7 breast cancer cells

Growth of MCF-7 cells has been shown to be mediated by E2 exposure via PI3K/Akt signaling pathways [43;44]. Similarly, superoxides and H_2O_2 have also been reported to activate PI3K/Akt signaling pathway which mediates survival and growth of exposed cells [18-20]. To determine whether E2 induced oxidants can activate PI3K/Akt signaling pathway and whether ROS scavengers or inhibitors of PI3K mitigates Akt phosphorylation, MCF-7 cells were either infected with biological antioxidants, or pretreated with chemical ROS scavengers (ebselen, NAC) prior to estrogen treatments as described in methods. WCL were resolved on 12% SDS-PAGE gel, and probed with appropriate antibodies. Data reveals that 100 pg/ml estrogen treatment induced phosphorylation of Akt which were inhibited by chemical or biological antioxidants as well as by LY294002, an inhibitor of PI3K which regulates Akt activations (Fig 4). These findings support the hypothesis that oxidant induced by estrogen exposures activatesignaling process that favors proliferation of estrogen responsive breast cancer cells.

Estrogen induced Akt activation phosphorylates NRF-1 which promotes growth of responsive cells

NRF-1 have been reported to be transactivated by Akt phosphorylation of its serine/threonine residue in response to growth factors and oxidative stress [45-47]. Transactivated NRF-1 are believed to mediate cellular responses to oxidative stress such as mitochondria biogenesis, restoration of redox homeostasis and induction of survival and proliferation mechanisms of cells [48].

Similarly, treatments of MCF-7 cells with 100 pg/ml estrogen for 45 minutes induced rapid phosphorylation of NRF-1 at its serine residues, compared to vehicle treated cells (Fig 5AI, B). NRF-1 phosphorylations were significantly diminished when cells were pre-treated with biological or chemical antioxidants prior to E2 treatments (Fig 5A II-IV, B). In addition, silencing of Akt_1, the Akt isoform implicated in mediating cell survival and growth [49-51] also reduced rapid phosphorylation of NRF-1 and anchorage independent growth of MCF-7 cells (Fig 6). This finding implies that estrogen induced redox signaling via Akt mediates rapid phosphorylation of NRF-1. Phosphorylated NRF-1 is believed to mediate cellular responses of breast cancer cells to estrogen exposure. These responses include regulations of mitochondria biogenesis, restoration of redox homeostasis and mediation of survival and growth of breast cancer cells exposed to different levels of estrogens.

Redox sensitive genes promote growth of estrogen responsive breast cancer cells

To show that NRF-1 mediates growth of breast cancer cells in vitro, NRF-1 was silenced in MCF-7 cells and then treated with with E2 and as control cells were cultured untreated for twenty one days. Colony formation, growth and viability of cells in soft agar assays were measured. Data reveals that estrogens increased colony formation and colony growth in null NRF-1 knockdown groups that was comparable to the untransfected cells (Fig 7B i-iv). However, estrogens failed to induce colony formation or colony growth of cells where NRF-1 was silenced. This result implies that NRF-1 is essential in the mediation of estrogen induced in vitro growth of MCF-7 cells (Fig 7B v,vi)). To further ascertain whether reduction in cell growth were as a result of cell death due to NRF-1 knockdown, cells from soft agar plates were counted for cell viability per 1000 cells using trypan blue exclusion assays. We found that while there were differences in cell death due to NRF-1 status, these differences were not significant enough to account for observed lack of colony formations or growth of cells in NRF-1 knockdown

versus sham knocked cells (data not shown). In addition, immunoprecipitation analysis of NRF-1 expressions and assessment of its phosphorylation status reveals that null silenced cells expresses increased NRF-1 proteins and phosphorylation in response to prolonged estrogen exposure (Fig-7AI,II) whereas silencing of NRF-1 inhibits its expressions or phosphorylation in response to chronic exposures to estrogens (Fig 7AIII). To confirm that NRF-1 are the mediators of these observations, we assessed the expressions of mtTFA, the gene that regulates mitochondrial biogenesis during periods of oxidative stress [52], which itself is transcriptionally regulated by NRF-1. Western blot analysis of cell lysates from sham knockdown vs NRF-1 knock down showed a correlation between silencing of NRF-1 and expressions of mtTFA (Fig 7). Taken together, these observations demonstrate that NRF-1 expressions and phosphorylation are essential in response to oxidants induced by chronic exposures of estrogen for in vitro growth of breast cancer cells.

Discussions

Estrogens are chemicals capable of promoting growth of breast cancer cells via ER and non-ER mediated pathways. Estrogens has also been reported to induce growth of breast cancer cells via mitochondria signaling mechanisms [2;3;34;53]. Here, we report that i) estrogen induced ROS mediates in vitro proliferation and growth of breast cancer cells; ii) the mechanism involves redox activation and phosphorylation of NRF-1; iii) over-expressions of biological ROS scavengers or treatments of cells with chemical antioxidants inhibits NRF-1 activation as well as reduction of growth of breast cancer cells.

The role of estrogen induced oxidants in breast carcinogenesis has long been established [2;3;53;54]. However, the role of these oxidants in the mediation of growth of cancer cells is controversial. In this study, we observed that treatments of MCF-7 cells with physiological levels of estrogens induced both intracellular ROS, and promotes in vitro growth of breast cancer cells. However, pre-treatment of cells with chemical or biological antioxidant prior to estrogen exposures inhibits ROS formation and in vitro growth of cells. These observations implies that the growth promoting properties of estrogen in malignant breast cancer cells are regulated in part by

estrogen induced oxidants as ROS scavengers abolish estrogen induced growth of breast cancer cells. This observation is in alignment with the antiproliferative properties of Resveratrol, Tamoxifen and vitamins which are also in part due to their antioxidant properties [55-58]. Likewise, it has also been demonstrated that over-expressions of MnSOD which converts superoxides to H_2O_2 or catalase which converts H_2O_2 to water, induces apoptosis and growth arrest of breast cancers cells [59-61]. However, there are caveats to this claim. For example; we observed that infecting cells with adenovirus over-expressing MnSOD at moi 50 or less increased estrogen induced intracellular ROS as well as increased proliferation and growth of breast cancer cells. However, infecting cells with moi 50 or greater induce lethal ROS production, as well as growth reduction or cells death. This observation indicates that the levels of estrogen induced oxidants determine whether breast cancer cell will survive and proliferate, or whether they will undergo cell death. Sarsour et al (2008) also indicated that low level MnSOD activities promoteproliferation of mouse embryonic fibroblasts (MEF) while high MnSOD activities induced growth arrest [62]. Hence, the roles of estrogen induced oxidants in regulating cell proliferation or cell death in breast cancer cells cannot be minimized. However, the mechanisms involved in this process are still unclear.

Cell signaling mechanisms that lead to proliferation and growth or apoptosis and cell death are complex and poorly understood. It has been reported that ROS mediates mitogenic activation of the PI3K/Akt signaling pathway which promotes survival and growth of exposed cells [63;64]. Likewise estrogens have been demonstrated to activate PI3K/Akt signaling pathways in endometrial and breast cancer cells, in an ER dependent and independent manner [65;66]. However, whether oxidants mediateestrogen induced activation of PI3K/Akt signaling in malignant breast cells and whether Akt activation is linked with growth of these cells is not clear. In our study, we observed that estrogen induced Akt activation in breast cancer cells were diminished by catalase over-expressions or by chemical ROS scavengers. Whether oxidant activation of Akt in estrogen exposed cells is linked with proliferation and growth regulation is not known. To address this link, we silenced Akt1, the Akt isoform known to be involved in regulating mammary tumorigenesis and growth of cancer cells [49;67] and

found that knocking down this gene diminished estrogen induced proliferation and growth of MCF-7 cells. These findings suggests that estrogen activation of Akt in breast cancer cells are ROS mediated and activation of Akt are essential in transducing cell signals that favors survival and growth of breast cancer cells. Therfore, it may be concluded that the growth promoting properties of estrogens in breast cancer cells are mediated separately by ER signaling mechanism and/or in combination with estrogen induced redox activations of Akt signaling pathways that favors proliferations and growth, over cell death.

Activated Akt promotes cell survival and growth via a number of mechanisms. For example, Akt phosphorylates and deactivates pro-apoptotic factors such as BAD (??), Caspase-9, and Forkhead transcription factors (FKHR) as well as other pro-survival transcription factors [68]. Other targets of activated Akt in response to oxidative stress includes NRF-1, a redox sensitive gene known to regulate transcription of genes involved in antioxidant responses as well as regulation of mitochondrial biogenesis [27;45;69]. Oxidant mediated phosphorylation of NRF-1 are thought to induce nuclear translocation and transcriptional regulation of various cellular response such as restoration of redox homeostasis, enhancement of cell survival and growth, as well as regulation of mitochondrial biogenesis [70]. Prolonged estrogen exposure has likewise been reported to up-regulate NRF-1 expressions via ER alpha (ERα) mediated mechanisms in MCF-7 cells which leads to increased mitochondria biogenesis in these cells [32]. Here, we report that chronic exposures of MCF-7 cells to estrogens not only up-regulates NRF-1 expressions, but also induces NRF-1 phosphorylation by Akt in a redox related manner. Treatment of cells with ROS modulators or silencing of NRF-1 fail to mediate estrogen induced NRF-1 expressions and phosphorylation, and inhibits growth of breast cancer cells. While ERα is believed to regulate estrogen induced NRF-1 expressions in MCF-7 cell line [32], our data indicates that estrogen induced oxidants are essential for NRF-1 expressions and phosphorylation. Upon phosphorylation, this transcriptions factor regulates expressions of mitochondria biogenesis, oxidative phosphorylation and mediates in oxidant induced survival and growth of breast cancer cells. These observation are in agreement with reports that has shown that NRF-1 is phosphorylated by oxidants in rat hepatoma cells which promotes survival and growth of these cells

during periods of oxidative stress [45]. Our data may also explain the observation that despite the high oxidative environment of breast cancer cells in vivo [11], these cells are able to survive, proliferate and grow as oppose to undergoing apoptosis and cell death. Activated NRF-1 may also underlie therapeutic failures and poor prognosis of breast cancer patients as oxidative stress are associated drug failures in a number of malignancies including breast cancer [71;72]. In addition, while it has been reported that estrogen and insulin induces proliferation and growth of MCF-7 and bovine mammary epithelium cells via NRF-1 activations [33], insulin, as with estrogens, are pro-oxidants [73;74] as well as activator of Akt signaling pathways [75;76] in a variety of cells types. It is therefore feasible that the pro-oxidative properties of these agents promote survival and growth of breast cancer cells through persistent expressions and phosphorylation of NRF-1.

The role of NRF-1 in cellular functions unrelated to mitochondria biogenesis and antioxidant responses are not new. Recently Cam et al, (2004) using genome-wide location analysis did find that NRF-1 associates with over 600 promoter regions of genes with diverse roles [77]. For example, while it has been revealed that NRF-1 cooperates with E2F targeted gene expression of human bone osteosarcoma and mammary gland adenocarcinoma to down-regulates proliferation [78;79] NRF-1 also binds to promoter regions of p115 genes of bovine mammary epithelium and MCF-7 cells to up-regulate proliferation in response to insulin and estrogen [33]. These opposing roles of NRF-1 indicate that this gene functions are diverse and not limited to regulation of mitochondria biosynthesis. It also indicates that estrogen induced growth in ER positive breast cancer cells are not limited solely to ER mechanism. The mitogenic effects of ROS induced by estrogen exposures cannot be discounted as a contributory factor in proliferation and growth of breast cancer cells.

In summary, estrogens are known to promote growth of breast cancer cells via ER related pathways and also elicit low levels of ROS which we have shown to be mitogenic in cancer cells (34). The mechanism of ROS induced growth in breast cancer involves redox activations and phosphorylation of NRF-1. Upon activation, NRF-1 regulates genes involved in redox responses as well as regulates genes that favor proliferation and growth of estrogen exposed cells. While the roles of ER mechanisms are not diluted by these findings, our study

however indicates that a level of redox status of MCF-7 cells exposed to estrogen is essential for NRF-1 expressions and growth regulation of breast cancer cells. Results from this study highlight the need to consider developing therapeutics target in the NRF-1 signaling mechanisms, and explore antioxidant based chemotherapies for treatments of breast cancer.

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Legends

Fig-1: **Estrogen induced ROS levels modulates proliferation of MCF-7 cells in vitro.**
A) cells infected with catalase at moi 50 and challenged with E2 had similar ROS productions and growth rate as E2 alone. Also, cells infected with MnSOD at moi 50 induced 60% more ROS than estrogen alone and induced 40% more DNA synthesis compared to estrogen alone. However, B) in cells infected with catalase at moi 200 and challenged with E2 produced 130% less ROS and 70% growth inhibition when compared to cells challenged with estrogen alone. In addition, cells challenged with MnSOD at moi 200 produced 300% more ROS and 100% less cell proliferation compared to estrogen treated group. All Cells treated with ebselen or NAC and subsequently challenged with estrogens had 150% less ROS production and cell proliferation compared to estrogen alone treated group. Data indicates that ROS modulators attenuates estrogen induced growth of MCF-7 cells.

Fig-2: **Differential effects of MnSOD overexpressions in MCF-7 cell growth in soft agar assay.** MCF-7 cells were infected with various concentrations of MnSOD overexpressing adenoviruses for 24hrs after which cells were detached and seeded in soft agar such that each 48 well had 5000 cells/well. Cells were incubated for 3 weeks with 100 pg/ml estrogens. A) Empty vector, B) Moi 5, C) moi 10, D) moi 50, E) moi 100, F) moi 200, G) moi 400. Data reveals that adenovirus expressing MnSOD infection of moi 50 or less promote estrogen induced growth whereas (B-D) moi 100 and above induced growth arrest in soft agar assay. Cells were fed weekly and images were acquired with hand held Nikon camera over an inverted microscope with 4x objective.

Fig-3: R**OS Scavengers inhibits E2 induced growth of MCF-7 in soft agar assay.** MCf-7 cells were infected with biological antioxidants for 24 hrs. in serum free media and allowed to recover for another 24 hrs in complete media. Cell were then detached and mixed 1:1 with 2x culture media containing all treatments and molten agarose such that each well contains 5000 cells. Cultures were allowed to incubate for 3-5wks with weekly feeding. i) DMSO, ii) 100pg/ml E2, iii) Catalase, iv) Catalase/E2, v) MnSOD, vi) MnSOD/E2, vii) Ebs, viii) Ebs/E2, I) At end of culture, images were acquired with a hand-held Nikon digital camera over an inverted microscope with 4x objective. B) Quadruplicate wells for each treatment was counted and expressed as mean +/- SD. Data shown are representative images from an experiment repeated three separate times. Data indicates that A) ROS modulators augments estrogen induced growth of MCF-7 cells. B) Colony formation count was normalized to colonies in vehicle treated cells.

Fig-4: **Estrogen induced activation of Akt is abrogated by ROS scavengers.** MCF-7 cells infected with adenovirus MnSOD or catalase cassettes at moi 100 for 24 hrs. in serum free media and allowed to recover in serum media for another 24 hrs. After 2 days of infections and 4 hrs before estrogen challenge, cells were treated either 40 μM ebselen or 1.0 mM NAC after which cells were challenged with estrogens. A) Data reveals ROS modulators and LY294002 (inhibitor of Akt activations) reduced estrogen activation of Akt. B) Band intensities quantified by Biorad GelDoc 2000 software and normalized to vehicle.

Fig 5: **ROS scavengers mitigates estrogen induced phosphorylations of NRF-1.** Cells were pre-infected or treated with 40 uM ebselen and processed for immunoprecipitations as described in methods. Anti NRF-1 was used to pull down NRF-1 protein in 750 μg whole cell lysate and eluate were resolved on 12 % SDS PAGE. 75 ug WCL were also resolved as loading control. A) Data reveals that biological or chemical ROS modulators inhibits NRF-1 phosphorylations. B) Band intensity was normalized and expressed as fold change from vehicle treatment. Panel 1) Non-infected cells treated with or without estrogen; Panel II) Catalase infected cells treated with or without estrogens; Panel III) MnSOD infected cells treated with or without estrogens; Panel IV), Ebselen treated cells with or without estrogens.

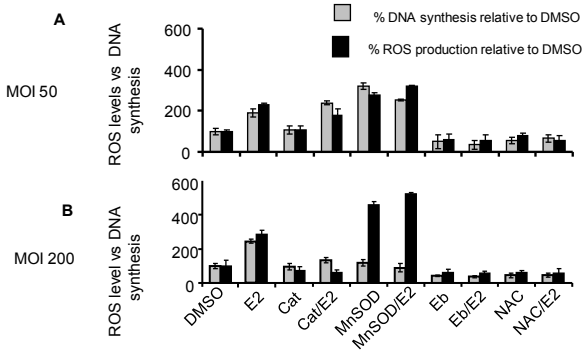
Fig-6: **Estrogen induced activations of Akt mediates growth of responsive malignant cells**. To determine whether estrogen induced Akt activation participate in growth of MCF-7 cells, cells were transfected with plasmids expressing shRNA for Akt1 or null plasmid as negative control as described in methods. Post transfection, cells were lysed for immunoprecipitations (IP) or immunoblots as described in methods. IP eluates were resolved in 12% SDS-PAGE and blots were probed with either anti phosphoserine, anti NRF-1, anti Akt or beta actin for control. Cells were also used for soft agar colony asay as previously described. Data reveals that A) estrogen induced NRF-1 serine phosphorylation in null transfected cells were diminished in Akt1 transfected cells. B) Akt1 silenced MCF-7 cells did not grow upon estrogen treatments. These data indicate therefore that Akt phosphorylation of NRF-1 is essential for estrogen-induced growth of MCF-7 cells. Panel I) Null transfected cells and treated with or without estrogen; Panel II) Aki1 silenced cells treated with or without estrogens.

Fig-7: **Silencing of NRF-1 attenuates growth of MCF-7 in response to estrogens.** siRNA for NRF-1 and its corresponding scrambled negative control (Null) were transfected into MCF-7 cells as previously described. Cells were subsequently used for immunoprecipitation and western blot assays (Panel A) as well as colony formation in soft agar colony assay (Panel B) as described in methods. To ascertain that NRF-1 knockdown modulates downstream genes, probed western blot with TFAM as this is one of the downstream targets of NRF-1 expression. Colonies formed were enumerated and normalized to vehicles (DMSO) treated cells. Data reveals that silencing of NRF-1 in MCF-7 cells inhibits estrogen-induced growth indicating that this transcription factor is essential for the growth of MCF-7 cells exposed to estrogen. A: i) DMSO, ii) E2, ii) null, iv) null/E2, v) NRF-1 kd, vi) NRF-1 kd/E2. B: Colony count in quadruplicate wells and expressed as mean +/- SD.

Fig-8: **Proposed Mechanism of E2 induced NRF-1 activation which mediate proliferation and growth:** Chronic exposure of mammary epithelial cells to estrogens generates sublethal levels of oxidative stress (production of ROS) via CYP450 metabolic activations as well as by induction of the mitochondria. These oxidants therefore mediate phosphorylation and activation of Akt signaling pathway as well as redox sensitive expressions of NRF-1. Activated Akt phosphorylates NRF-1 to mediate its nuclear translocation. In the nucleus, NRF-1 binds to 'NRF-1 responsive elements' (NRF-1RE) and regulates cadre of genes expressions involved in mitochondria biogenesis as well as genes involved in Apoptosis, survival and proliferations and growth in subsets of cells.

Estrogen induced ROS levels modulates proliferation of MCF-7 cells in vitro

Fig 1

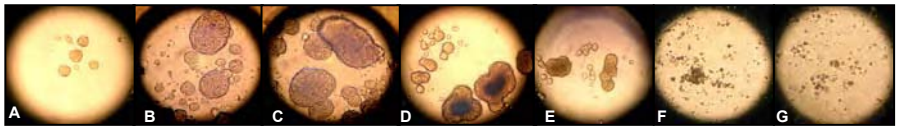


MCF-7 cells were seeded overnight at 3500 cells/well in 96 well plate for cell proliferation assays and 1.0x10^4cells/well for ROS production assays. 18 hrs post seeding, cells were infected with Adenovirus over-expressing MnSOD or Catalase at either moi 50 or 200 in serum free media. Other group of cells was treated with either 40µm ebselen or 1mM NAC respectively, diluted in serum free media. Cells were cultured for 48hrs. For ROS assays, cells were pretreated for 4hrs before assay with ebselen and NAC after which 10µM DCF were loaded onto the cells for 20 mins, then cells were challenged with 100 pg/ml estrogen and readings were taking every 5mins on Tecan plate reader. For cell proliferation assays, 48hrs post infections, wells were challenged with 100 pg/ml estrogen and allowed to incubate for additional 18hrs after which BrdU assay were carried out with Roche BrdU assay kit. ROS and BrdU data are expressed as means of 3 plate x 4 wells/plate, +/- SD.

Data reveals that A) cells infected with catalase at moi 50 and challenged with E2 had similar ROS productions and growth rate as E2 alone. Also, cells infected with MnSOD at moi 50 induced 60% more ROS than estrogen alone and induced 40% more DNA synthesis compared to estrogen alone. However, B) in cells infected with catalase at moi 200 and challenged with E2 produced 130% less ROS and 70% growth inhibition when compared to cells challenged with estrogen alone . In addition, cells challenged with MnSOD at moi 200 produced 300% more ROS and 100% less cell proliferation compared to estrogen treated group. All Cells treated with ebselen or NAC and subsequently challenged with estrogens had 150% less ROS production and cell proliferation compared to estrogen alone treated group. Data indicates that ROS modulators attenuates estrogen induced growth of MCF-7 cells.

Differential effects of MnSOD overexpressions in MCF-7 cell growth in soft agar assay

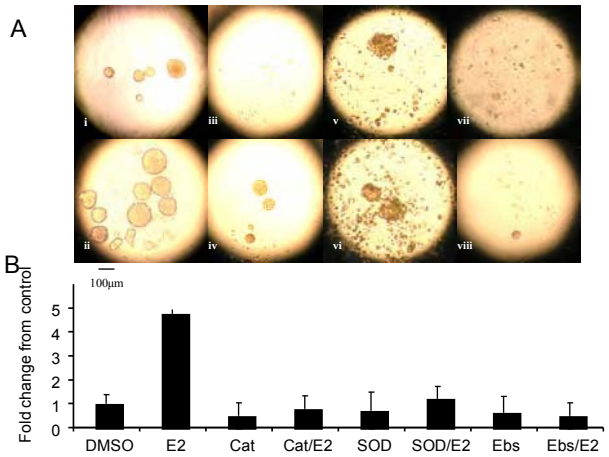
Fig 2



MCF-7 cells were infected with various concentrations of MnSOD overexpressing adenoviruses for 24hrs after which cells were detached and seeded in soft agar such that each 48 well had 5000 cells/well. Cells were incubated for 3 weeks with 100 pg/ml estrogens. Cells were fed weekly and images were acquired with hand held Nikon camera over an inverted microscope with 4x objective. A) Empty vector, B) Moi 5, C) moi 10, D) moi 50, E) moi 100, F) moi 200, G) moi 400. Data reveals that adenovirus expressing MnSOD infection of moi 50 or less promotes estrogen induced growth whereas (B-D) moi 100 and above induced growth arrest in soft agar assay.

ROS Scavengers inhibits E2 induced growth of MCF-7 in soft agar assay

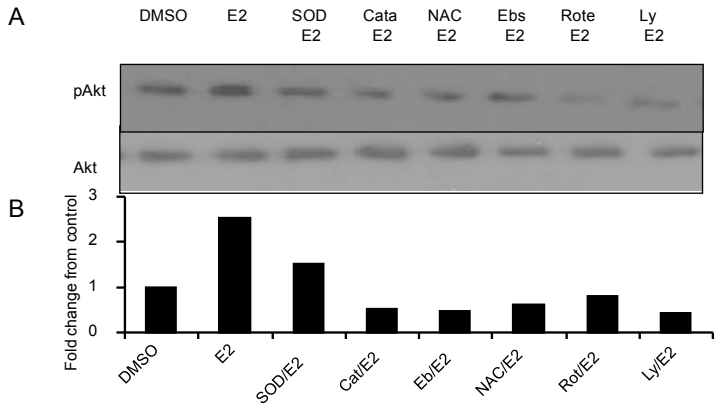
Fig 3



To determine whether ROS scavengers modulates estrogen induced growth of MCF-7 cells, cells were infected with biological antioxidants for 24 hrs in serum free media and allowed to recover for another 24 hrs in complete media. Cell were then detached and mixed 1:1 with 2x culture media containing all treatments and molten agarose such that each well contains 5000 cells. Cultures were allowed to incubate for 3-5wks with weekly feeding. i)DMSO, ii) 100pg/ml E2, iii) Cata, iv) Cata/E2, v) MnSOD, vi) MnSOD/E2, vii) Ebs, viii) Ebs/E2, I) At end of culture, images were acquired with a hand-held Nikon digital camera over an inverted microscope with 4x objective. B) Quadruplicate wells were counted and expressed as mean +/- SD. Data shown are representative images from an experiment repeated three separate times. Data indicates that A) ROS modulators augments estrogen induced growth of MCF-7 cells. B) Colony formation count normalized to colonies in vehicle treated cells.

Estrogen induced activation of Akt is abrogated by ROS scavengers

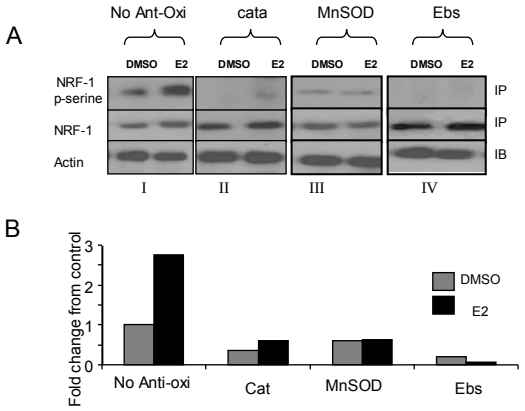
Fig 4



To determine whether ROS modulates Akt activations in MCF-7 cells treated with estrogens, MCF-7 cells were infected with adenovirus over-expressing catalase or MnSOD at moi 100 for 24hrs in serum free media and allowed to recover in serum media for another 24 hrs. 2 days post infections and 4 hrs before estrogen challenge, cells were treated either 40 uM ebselen or 1.0 mM NAC after which cells were challenged with estrogens. A) Data reveals ROS modulators and LY294002, inhibitor of Akt activations, reduced estrogen activation of Akt. B) Band intensity were obtained by Biorad GelDoc 2000 software and normalized to vehicle.

ROS scavengers mitigates estrogen induced phosphorylations of NRF-1

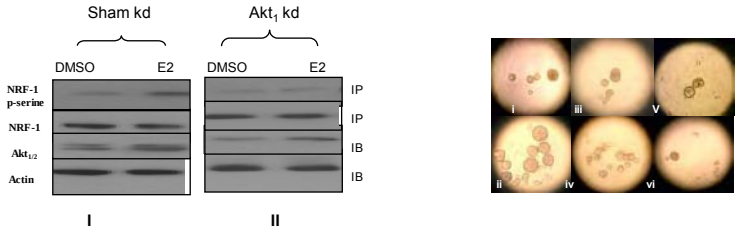
Fig 5



To determine whether ROS modulators attenuate estrogen induced NRF-1 expressions and serine phosphorylation, cells were pre-infected or treated with 40 uM ebselen as previously described and processes for immunoprecipitations as described in methods. NRF-1 were used to pull down NRF-1 protein in 750 ug whole cell lysate and eluate were resolved on 12 % SDS PAGE. 75 ug WCL were also resolved as loading control. A) Data reveals that biological or chemical ROS modulators inhibits NRF-1 phosphorylations. B) Band intensity were normalized and expressed as fold change from vehicle treatment. Panel 1) Non-infected cells treated with or without estrogen; Panel II) Catalase infected cells treated with or without estrogens; Panel III) MnSOD infected cells treated with or without estrogens; Panel IV), Ebselen treated cells with or without estrogens.

Estrogen induced activations of Akt mediates growth of responsive malignant cells

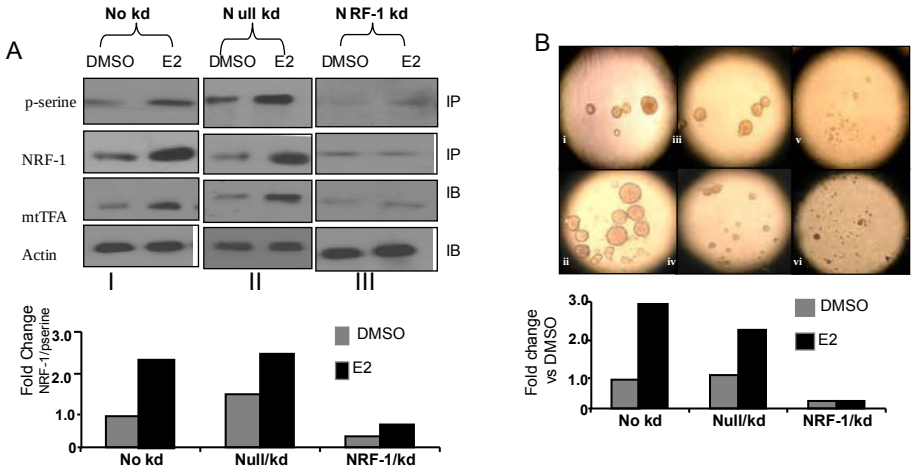
Fig 6



To determine whether estrogen induced Akt activation participates in growth of MCF-7 cells, cells were transfected with plasmid expressing shRNA for Akt1 or null plasmid as negative control as described in methods. Post transfection, cells were lysed for immunoprecipitations or immunoblots as described in methods. IP eluates were resolved in 12% SDS-PAGE gel and blots were probed with either anti phosphoserine, anti NRF-1, anti Akt or beta actin for control. Some cells were also used for soft agar colony asay as previously described. Data reveals that A) estrogen induced NRF-1 serine phosphorylation in null transfected cells were diminished in Akt1 transfected cells. B) Akt1 silenced MCF-7 cells did not grow upon estrogen treatments. These data indicates therefore that Akt phosphorylation of NRF-1 are essential estrogen induced growth of MCF-7 cells. Panel I) Null transfected cells and treated with or without estrogen; Panel II) Aki1 silenced cells treated with or without estrogens.

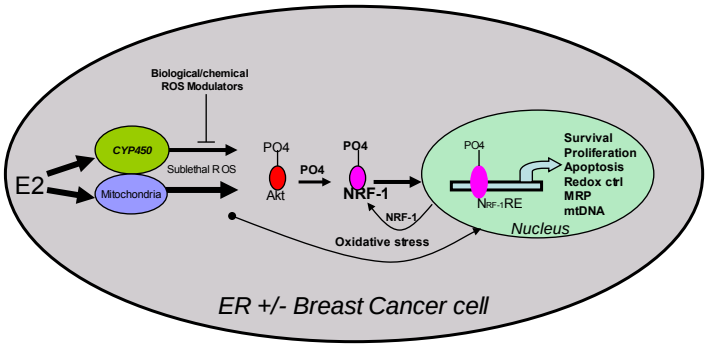
Fig 7

Silencing of NRF-1 attenuates growth of MCF-7 in response to estrogens



To determine if NRF-1 participates in growth promotion in estrogen treated MCF-7 cells, we transfected MCF-7 cells with siRNA for NRF-1 and its corresponding scrambled negative control (Null) into MCF-7 cells as previously described. Cells were subsequently used for immunoprecipitation and western blot assays (Panel A) as well as colony formation in soft agar colony assay (Panel B) as described in methods. To ascertain that NRF-1 knockdown modulates downstream genes, probed western blot with mtTFA as this is one of the downstream targets of NRF-1 expression. Colonies formed were enumerated and normalized to vehicles (DMSO). Data reveals for first time that silencing of NRF-1 in MCF-7 cells inhibits estrogen induced growth indicating that this transcription factor is essential to growth of MCF-7 cells exposed to estrogen. A: i) DMSO, ii) E2, ii) null, iv) null/E2, v) NRF-1 kd, vi) NRF-1 kd/E2. B: Colony count in quadruplicate wells and expressed as mean +/- SD.

Proposed Mechanism of E2 induced NRF-1 Activation which mediate Survival, Proliferation and growth of Breast cancer cells



Chronic exposure of mammary epithelial cells to estrogens generates sublethal levels of oxidative stress via CYP450 metabolic activations as well induction of the mitochondria. These oxidants therefore mediates phosphorylates and activation of Akt signaling pathway as well as redox expressions of NRF-1. Activated Akt phosphorylates NRF-1 and mediates it nuclear translocation.
In the nucleus, NRF-1 binds to 'NRF-1 responsive elements' (NRF-1RE) and regulates cadre of genes expressions involved in mitochondria biogenesis as well as genes involved in Apoptosis, survival and proliferations and growth in subsets of cells.

4. Two additional Manuscripts are under preparation

5. List of Personnel received pay from research

Deodutta Roy –PI
Quentin Felty –Investigator
Yong Ping Zou - Postdoctoral fellow
Brian Kunkle –Graduate Research Assistant
Amy Kennedy – Graduate Research Assistant
Victor Okoh – Graduate Research Assistant